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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C07K 15/00, G01N 33/68 — A1

(11) International Publication Number: WO 92/18535

(43) International Publication Date: 29 October 1992 (29.10.92)

(21) International Application Number: PCT/US92/02888

(22) International Filing Date: 9 April 1992 (09.04.92)

(30) Priority data:
682,177 9 April 1991 (09.04.91) US

(60) Parent Application or Grant
(63) Related by Continuation
US 682,177 (CIP)
Filed on 9 April 1991 (09.04.91)

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(81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, KR, LU (European patent), MC (European patent), NL (European patent), SE (European patent), US.

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: ENDOMETRIAL ANTIGEN, COMPOSITION, TEST KIT AND METHOD FOR ENDOMETRIAL ANTIBODY DETERMINATION

(57) Abstract

A plurality of protein antigen fragments have been isolated from the cytoplasm of epithelial adenocarcinoma cells. The protein antigens are useful in the detection of endometrial antibodies which are indicative of endometriosis. The antigens can be attached to water insoluble supports or detectably labeled to form reagents. Detection of endometrial antibodies is accomplished by reacting the antigen with the antibodies in a specimen sample followed by detection of the resulting complex. The antigens can be supplied as a buffered composition in a diagnostic test kit.

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ENDOMETRIAL ANTIGEN, COMPOSITION, TEST KIT AND METHOD FOR ENDOMETRIAL ANTIBODY DETERMINATION

Field of the Invention

This invention relates to the detection of endometriosis, and to protein antigenic fragments, reagents, diagnostic test kits and compositions useful therein.

Background of the Invention

Endometriosis is a disease state in which tissues resembling the uterine mucous membrane, or endometrium (located in the lining of the uterus), multiply in other parts of the body, such as in the abdominal cavity. This disease is a significant problem in gynecology. The presence of the abnormal tissues can cause abdominal bleeding, adhesions, dysmenorrhea and particularly infertility.

Currently, a preliminary diagnosis of endometriosis is generally made based on a patient's history of infertility, unexplained pelvic pain or other known symptoms. Confirmation is carried out using a surgical procedure termed laparoscopy to obtain a sample of tissue for biopsy. This is a procedure which is unpleasant as well as having the usual dangers associated with invasive procedures.

It has been reported that antibodies to normal endometrial tissues have been found in the serum of patients with endometriosis (see publications noted in EP-A-0 387 027, published September 12, 1990). Various antigens have been speculated as immunologically related to the endometrial antibodies found in the serum specimens.

In EP-A-0 387 027, endometrial antigens having various molecular weights were described as isolated from cultures or culture media obtained from several epithelial carcinoma cell lines. Monoclonal antibodies and immunological reagents directed to the

antigens are also described. The antibodies and antigens were then used to detect endometrial antibodies in patient specimens using various immunological procedures.

It would be desirable to have additional antigens which could be used in a sensitive and accurate assay for endometrial antibodies.

Summary of the Invention

The present invention provides a protein antigen isolated from the cytoplasm of epithelial adenocarcinoma cells, the antigen selected from the group consisting of:

a. a fragment having a molecular weight of from about 63 to about 67 kilodaltons,

b. a fragment having a molecular weight of from about 33 to about 37 kilodaltons,

c. a fragment having a molecular weight of from about 40 to about 44 kilodaltons

d. a fragment having a molecular weight of from about 31 to about 35 kilodaltons, and

e. a fragment having a molecular weight of from about 57 to about 64 kilodaltons.

These antigens can be provided individually or in admixture in a buffered composition useful for detecting the presence of endometrial antibodies.

This invention also provides an endometrial antibody capture reagent comprising one or more of the protein endometrial antigens described above attached to a water insoluble support.

Additionally, one or more of the antigens can be detectably labeled to provide water soluble endometrial antibody detection reagents.

The antigen or any of the reagents described above can be provided in a diagnostic test kit along with an anti-human antibody reactive with an endometrial antibody.

A method for determining endometriosis comprises:

A. contacting a specimen suspected of containing endometrial antibodies with a protein antigen as described above, and

B. detecting any resulting complex of the antigen with the endometrial antibodies as an indication of the endometrial antibodies in the specimen.

The present invention provides an advantageous means for detecting endometriosis without the need for invasive laparoscopy. This result is achieved using novel protein antigen fragments isolated from the cytoplasm of epithelial adenocarcinoma cells to detect the presence of endometrial antibodies in a patient specimen, such as serum. Sensitive detection of the antibodies can be carried out using various assay formats, as described below.

Brief Description of the Figure

The FIGURE is a photographic image of several nitrocellulose strips used in an immunoblot assay, as described in more detail in Example 5 below.

Detailed Description of the Invention

The antigens of this invention are identified generally by molecular weight in kilodaltons. They are identified in a narrow range of molecular weights since it is standard in the art to have some inherent inaccuracy (about 10%) in electrophoretic methods for molecular weight determination. Some of the antigens have also been characterized by isoelectric point (pI).

The antigens are generally protein fragments isolated from larger proteins found in the cytoplasm of human epithelial adenocarcinoma cells. Included among such cells are endometrial, breast and ovary cells. The antigen fragments identified herein can be isolated from various cell lines, and may have varying amino

acid compositions even though the molecular weight is the same. Also contemplated as equivalents of the naturally occurring antigens isolated from cells are what are termed "immunological equivalents" which are peptides which have the same molecular weight, isoelectric point and immunological reactivity with the antibodies of interest.

Representative isolated fragments of this invention are listed in Table I below, and can be used singly or in mixtures in the practice of this invention.

TABLE I

Cell Line	Antigen Fragment	Molecular Weight (kD)
RL95-2 (ATCC CRL-1671)	A	63-67
AN3CA (ATCC HTB-111)	B_1	63-67
	B_2	33-37
	B_3	40-44
	B_4	59-64
HEC1A (ATCC HTB-112)	C_1	63-67
	C_2	33-37
	C_3	40-44
	C_4	59-64
KLE (ATCC CRL-1622)	D	31-35
T47D (ATCC HTB-133)	E_1	63-67
	E_2	33-37
	E_3	40-44
	E_4	59-63
	E_5	57-59

CAOV3 (ATCC HTB-75)	F_1	63-67
	F_2	33-37
	F_3	40-44
	F_4	59-64

It is preferred to use the 33-37 kD, 40-44 kD and 57-59 kD fragments noted above, individually or in a mixture, with the mixture of two or more fragments being most preferred.

Endometrial antigens can be isolated by affinity chromatography of extracts of epithelial tissue (such as endometrial tissue) which has been subjected to extraction reagents such as detergents. Antibodies (monoclonal or polyclonal) specific to the antigens can be used in the chromatography process. The treatment of the tissue extracts by column purification can be carried out using standard procedures, for example, those described by Davis et al, Canc. Res. 46, pp. 6143-6148 (1986). By "isolated" is meant that the protein antigen fragment is in an at least partially purified state compared to its natural state in the cytoplasm of the epithelial adenocarcinoma cells, or in any other human fluid or tissue specimen. Various techniques besides those described herein can be used to isolate the individual antigen fragments including electroelution from gels as described, for example, by Harrington, Methods of Enzymology 182, pp. 488-495 (1990).

In a preferred process, the antigens can be obtained from tissue culture cells such as cultures of epithelial adenocarcinoma cell lines such as those mentioned in EP-A-0 387 027. Representative useful cell lines are on deposit with the American Type Culture Collection (Rockville, Maryland), namely: cell lines HEC1A (ATCC HTB-112), AN3CA (ATCC HTB-111), RL95-

2 (ATCC CRL-1671), KLE (ATCC CRL-1622), T47D (ATCC HTB-133) and CAOV3 (ATCC HTB-75).

The general procedure for isolating the antigens from a cell line is as follows: The cells are grown in the recommended medium to greater than 90% confluency, followed by homogenization in buffered saline solution or a solution of tris(hydroxymethyl)-aminomethane, sucrose and protease inhibitor. For extracts in buffered saline solution, particulate material is removed by centrifugation and the supernatant concentrated. In a preferred embodiment, for extracts in the sucrose solution, particulate materials are removed by centrifugation and the supernatant is spun at 100,000 x gravity for 1 hour at 4°C, then concentrated. Extracts (that is, the cytoplasmic fraction) are then resolved using SDS-PAGE electrophoresis for an appropriate time and voltage in an appropriate buffered system. The resulting proteins are then transferred to nitrocellulose using standard immunoblotting techniques described, for example, by Stott, J. Immun. Methods 119, pp. 153-187 (1989). More details about this procedure are provided in Example 1 below. All the materials used in the extraction procedure are commercially available.

The isolated antigen or mixture thereof can be supplied in a buffered composition for use in various immunological methods. The composition is generally buffered to a pH of from about 6 to about 8 using one or more suitable buffers such as phosphate buffered saline solution, tris(hydroxymethyl)amino-methane, glycine, 3-(N-morpholino)propanesulfonic acid, borates, and others known in the art, [for example, Good et al, Biochem., 5, 467 (1966)], most of which are commercially available. The amount of antigen in such a composition can vary widely depending upon its intended use. The isolated antigen fragments can be

used in crude form (that is, in admixture with extraneous cellular materials) or at various levels of purification.

The antigens described herein can also be provided as detectably labeled water soluble (or water suspendible) reagents which have an appropriate label moiety coupled thereto. Useful labels include those directly detectable, such as radioisotopes, chromogens, fluorogens, suspendible magnetic particles, suspendible dyed polymeric particles, chemiluminescent moieties, bioluminescent moieties, phosphors and others known in the art. Labels which are indirectly detectable through reaction with additional reagents include enzymes, dye-formers and others known in the art (for example, in EP-A-0 387 027). Particularly useful labels include radioisotopes and enzymes. Useful enzyme labels include peroxidase, alkaline phosphatase, urease, glucose oxidase, beta-galactosidase and others readily apparent to one skilled in the art. Peroxidase and alkaline phosphatase are preferred.

The label moieties can be coupled to antigen fragments using standard technology described, for example, in US-A-4,302,438, Marchalonis, Biochem. J., 113, pp. 299-305 (1969) Hnatowich et al, J.Immunol. Methods, 65, pp. 147-157 (1983) and Science, 220, pp. 613-615 (1983) for radiolabeling, and Yoshitake et al, Eur.J.Biochem., 101, 395 (1979), Pesce et al, Clin.Chem., 20, pp. 353-359 (1974), US-A-4,302,438, US-A-4,376,110 and US-RE-31,006 and references mentioned therein, for example, for labeling with enzymes. Antigens can be coupled to magnetic or magnetizable particles using the teaching of, for example, US-A-4,795,698. Chemiluminescent moieties can be coupled to antigens according to the teaching of, for example, US-A-4,380,580. Dyed or fluorescent particles are useful as labels and can be

attached to antigen according to US-A-4,259,313, EP-A-0 208 556 and EP-A-308 235. Fluoroscein or other fluorescent moities can be attached as a label using known procedures.

The antigen can also be labeled with a specific binding moiety that is not specific for endometrial antibodies. Such moieties include avidin, biotin, a lectin, a sugar and others readily apparent to one skilled in the art. The moiety would be reactive with its corresponding receptor which can be labeled with an enzyme radioisotope or other moiety as described above. For example, if the antigen is labeled with biotin, the corresponding receptor, avidin, can be coupled to an enzyme. Such labeling techniques are described, for example, in US-A-4,496,654, EP-A-0 201 079 and EP-A-0 370 694.

For particles used in this reagent to be water suspendible, normally they are less than about 1 mmeters in size so that they stay suspended in water for at least 3 hours with little or no agitation.

The antigen of this invention can also be coupled with water insoluble supports to provide endometrial antibody capture reagents for complexing with the endometrial antibody, thereby "capturing" the antibody. Any useful support can be used as long as it is not readily suspendible in water (unlike the reagents described above) and does not interfere with the antibody-antigen reaction or any other reactions necessary for detection of that immunological reaction. Useful supports include particles of organic and inorganic polymers, glass, ceramics, silica gel, metals, metal oxides, filters of paper, glass, matted fibers and particulate structures, microporous polymeric filters, gels, microtiter plates, test tubes, test cups, vials and others readily apparent to one skilled in the art. The particles are generally

greater than about 0.05 m meters in diameter. Useful materials for such supports would also be apparent to one skilled in the art particularly in view of the teaching in EP-A-0 387 027. The antigens can be attached to such materials by adsorption or other non-covalent means (see for example US-A-4,528,267) or covalent means using techniques generally known, including those described above for coupling particulate labels to antigen, and others described by Chibata, Immobilized Enzymes, Halsted Press: New York (1978) and Cautrecasas, J.Bio.Chem., 245, 1059 (1970). Thus, coupling can be directly to the support through reactive groups or ionic bonds, or through coupling moieties or proteins as is known in the art. For example, the antigens can be coupled to microtiter plates by non-specific adsorption or covalent coupling to a reactive group on the plate.

Antibodies to the antigens of this invention can be developed using standard technology. For example, polyclonal antibodies can be prepared using suitable mammals, such as goats, monkeys, rabbits, guinea pigs and horses. The resulting antisera can be purified using conventional affinity chromatography such as described by Mishell et al, Selected Methods in Cellular Immunology, San Francisco, Freeman (1980).

Non-human monoclonal antibodies can also be prepared using the standard method of Köhler et al, Nature, 256, pp. 495-497 (1975) involving the use of hybridomas prepared from immunized mice or rats to produce suspended spleen cells. Suitable hybridomas are available from either commercial sources or various cell culture collections including the ATCC.

Other types of antibodies, including human monoclonal antibodies specific to the antigen and antiidiotypic antibodies can be prepared using the procedures described in more detail in EP-A-0 387 027.

The antigens of this invention are useful for the detection (that is, measuring the presence, amount or both) of endometrial antibodies found in human body fluids, such as whole blood, blood serum, suspensions of endometrial tissues, peritoneal fluid and uterine fluid or secretions. Preferably, the antibodies are detected in blood serum. These antibodies are generally identified as human IgG type antibodies although IgA type antibodies may also be present.

Detection of endometrial antibodies can be carried out using a variety of immunological methods, all of which are generally well known in the art as involving the preferential binding of the antigens of this invention with the corresponding endometrial antibodies. Such methods include, but are not limited to, competitive binding assays, enzyme-linked immunosorbent immunoassays (ELISA), radioimmunoassays (RIA), immunometric assays (sandwich), immunoblots, agglutination assays, light scattering assays and ultrasonic probe assays.

Immunoblots can be carried out using standard procedures described, for example, by Stott (noted above). Generally, the antigen is transferred to an immunoblot medium such as nitrocellulose (which is preferred), nylon or polyvinylidine difluoride, nonspecific sites are blocked with appropriate materials, and the patient sample is brought into contact with the medium for a sufficient period of time and temperature for antibodies in the sample to complex with antigen in the medium. Following washing, the complex in the medium can be contacted with detectably labeled antigen which can "sandwich" the antibodies, or with detectably labeled anti-human antibodies which are reactive with the endometrial antibodies. A representative immunoblot is described in Example 5 below, and the results shown in the Figure.

Light scattering assays are useful to detect endometrial antibodies using the antigens of this invention according to the teaching of EP-A-0 387 027.

Competitive binding assays generally involve contacting the specimen suspected of containing endometrial antibodies with the antigen and a known quantity of labeled endometrial antibodies. The labeled and unlabeled antibodies compete to complex with the antigen, and the amount of detectable signal from the complex is inversely proportional to the amount of unlabeled antibodies in the specimen. The labeled antibodies can be prepared using materials and procedures described generally above for labeled antigen. Further details of such assays can be obtained by consulting the considerable literature in this art, including US-A-3,654,090.

Another type of immunoassay is what is known as an immunometric or sandwich assay in which the targeted endometrial antibodies are "sandwiched" between specific binding materials. In one embodiment, the specific binding materials both comprise endometrial antigen, one being detectably labeled (that is a detection reagent), and the other being a capture reagent as described above. In another embodiment one specific binding material in the sandwich can be either a capture or detection reagent comprising an antigen as described herein, and the other is a capture or labeled anti-human antibody reactive with the endometrial antibodies. The anti-human antibodies are advantageously labeled with a fluorogen, enzyme or radioisotope. Where the anti-human antibody is labeled with an enzyme, the assay is known as an ELISA. Specific details about such assays are well known in the art, including US-RE-32,696, US-A-4,376,110 and US-A-4,486,530. Anti-human antibodies reactive with the

endometrial antibodies can be prepared using standard techniques. Many are commercially available.

Endometrial antibodies can be isolated from serum or other patient specimens by transferring an antigen fragment of this invention to nitrocellulose, contacting the immobilized antigen with the patient sample to complex the endometrial antibodies, and eluting the antibodies off the nitrocellulose using a high salt or low pH solution according to standard procedures.

In many of the assays described above, various wash solutions, blocking solutions, and dye-providing solutions (if the label is an enzyme or other chemical requiring further reaction for detection) may be needed. The details of such materials would be readily apparent to one skilled in the art having relevant publications at hand. Obviously, the specific reagents used would be dependent upon the form of assay and labels used therein.

Patient samples, such as serum samples, can be diluted if desired with water, buffer or suitable diluents commercially available for that purpose. Particularly useful diluent compositions are described in EP-A-0 337 785 (published October 18, 1989).

The assays can be carried out in appropriate equipment or test devices. Immunoblots, for example, are carried out using appropriate media, such as nitrocellulose strips. Competitive binding and sandwich assays can be carried out using microtiter plates having a multiplicity of test wells, test tubes, test slides, disposable test devices such as those described in US-A-3,825,410, US-A-3,888,629, US-A-3,970,429, US-A-4,446,232, US-A-4,870,007, US-A-4,921,677 and US-A-4,948,561, and other containers readily apparent to one skilled in the art. Preferred test devices include micro-titer plates and disposable

test devices (commercially available in SURECELL™ test kits marketed by Eastman Kodak Company) having microporous membrane disposed therein for separating complexed materials from uncomplexed materials.

The antigens, compositions or reagents of this invention can be supplied individually or as part of a diagnostic test kit. Such kits may include the compositions and reagents (noted above) used in particular assays as well as the necessary instructions, test devices, specimen handling equipment for assaying one or more specimens. Preferably, the kit includes the antigen (or mixture thereof), labeled anti-human antibodies reactive with the endometrial antibodies, and a means for detecting the resulting immunological reaction. The detecting means can be a test device, microtiter plate, a dye-providing composition or others known in the art, or a combination thereof.

The following examples are presented here to illustrate the practice of this invention. They are not meant to be limiting in the scope or specific embodiments. Unless otherwise indicated, the percentages are by weight.

Example 1: Isolation of Endometrial Antigens

The following procedure and materials were used to isolate several antigen fragments using various epithelial adenocarcinoma cell lines.

Six cell lines: HEC1A (ATCC HTB-112), AN3CA (ATCC HTB-111), RL95-2 (ATCC CRL-1671), KLE (ATCC CRL-1622), T47D (ATCC HTB 133) and CAOV3 (ATCC HTB-75) were obtained from the American Type Culture Collection (Rockville, Maryland).

Each cell line was treated in the following manner: it was grown in recommended media (for example, commercially available McCoy's media for HEC1A) to greater than 90% confluency (at this level, there is

confidence that one or more of the antigens are present). The resulting cells were homogenized in a solution of tris(hydroxymethyl)aminomethane buffer (50 mmolar, pH 7.4), sucrose (250 mmolar) and protease inhibitor (a mixture of 0.5 µg/ml of leupeptin, 0.7 µg/µl of pepstatin, 372 µg/ml of EDTA Na_2 and 2 µg/ml of aprotinin available from Boehringer-Mannheim or Sigma Chemical) for 2 minutes at 4°C using a mechanical homogenizer. Cellular debris, including nuclei, mitochondria and unlysed cells, was removed by centrifugation, followed by ultracentrifugation at 100,000 x gravity for 1 hour at 4°C to remove all unwanted cellular debris and leaving the contents of the cytoplasm. The supernatant was then concentrated using a Centricell concentrator (30,000 normal molecular weight limit, Polysciences, Warrington, Pa.) to about 0.5-10 mg/ml protein.

The extracts were resolved using SDS-PAGE electrophoresis with a 10% uniform polyacrylamide reducing gel in a buffer solution of tris(hydroxymethyl)aminomethane buffer (25 mmolar, pH 8.5), glycine buffer (200 mmolar), sodium dodecylsulfate (0.1%) and sodium acetate (100 mmolar) for 3-4 hours, increasing the voltage to 400 volts.

Isoelectric point (pI) was determined by two-dimensional electrophoresis. The cell lysate proteins, prepared as described above, were first separated by isoelectric point using isoelectric focusing in the first dimension, and then the electrofocused proteins were separated according to molecular weight by SDS-PAGE electrophoresis in the second dimension. The proteins were blotted to nitrocellulose as described in Stott, J.Immun. Methods, 119, pp. 153-187, (1989). The blotted protein was probed with patient serum. Sera believed to contain endometrial antibodies (as determined by indirect immunofluorescence microscopy)

and as being from patients known to have endometriosis (as determined by laparoscopy) were tested. Sera known to be negative for endometrial antibodies using similar techniques were also tested as negative controls. Reactive proteins were developed using a standard detection system. The positive serum antigens (which are the 63-67 kD, 40-44 kD, 33-37 kD, 57-59 kD and 59-64 kD antigens) were compared to the negative serum antigens (which are common to negative and positive serum: which may include 30-32 kD, 18-22 kD, 47-50 kD and 28-30 kD antigens). Antigens unique to the positive serum were then noted and the molecular weights and isoelectric points calculated. In other words, the antigens were identified by subtracting the common bands on the immunoblots from the bands shown on the immunoblot for the positive serum sample.

The molecular weight of each fragment was determined by comparing measured distances (cm) of target antigens to the measured distances (cm) of a resolved mixture of standard SDS-PAGE low molecular weight protein markers. Molecular weights were determined and expressed in kilodaltons (kD) using a polynomial curve fit calculated using CRICKETGRAPH™ software (available from Cricket Software, Inc.), and EXCEL™ software (available from Microsoft). Both programs were run on a Macintosh 2 computer.

The isolated antigen fragments and available data are listed in Table II below. The molecular weight of each fragment is listed as a narrow range because the exact value is difficult to determine using known procedures and a 10% variation is generally accepted in the art.

T A B L E II

Antigen Fragment	Cell Line Source	Molecular Weight (kD)**	Isoelectric Point @

A	RL95-2	63-67	NA*
B_1	AN3CA	63-67	NA
B_2	AN3CA	33-37	NA
B_3	AN3CA	40-44	NA
B_4	AN3CA	59-64	NA
C_1	HEC1A	63-67	4.5
C_2	HEC1A	33-37	5.8
C_3	HEC1A	40-44	4.6
C_4	HEC1A	59-64	6.0
E_1	T47D	63-67	4.5
E_2	T47D	33-37	NA
E_3	T47D	40-44	4.6
E_4	T47D	59-64	NA
E_5	T47D	57-59	NA
F_1	CAOV3	63-67	4.5
F_2	CAOV3	33-37	5.5
F_3	CAOV3	40-44	4.6
F_4	CAOV3	59-64	6.0

*NA = not available

** kD = kilodaltons

@ values reflect plus or minus 1.0

Example 2: Buffered Compositions of Antigens

One or more of the antigens were added to tris(hydroxymethyl)aminomethane buffer (pH 8.3) to form a crude buffered composition of this invention. These compositions can be stored in suitable containers, for example in test kits, until their use or immobilization on solid supports for use in assays.

Example 3: Preparation of Detectably Labeled Endometrial Antibody Reagent

This is a prospective example of how a detectably labeled endometrial antibody reagent of this invention can be prepared. This reagent would comprise an endometrial antigen fragment (isolated as described

above) which is labeled with 5-dimethylamino-naphthalene-1-sulfonyl chloride, a fluorescent moiety.

The reagent could be prepared by adding a 3- to 5-fold molar excess of 5-dimethylaminonaphthalene-1-sulfonyl chloride to isolated antigen fragment in tris(hydroxymethyl)aminomethane buffer (25 mmolar, pH 8.3). Before the addition, the chloride is dissolved in acetone equal to 1% of the final volume. Reaction would proceed for 1 to 2 hours at room temperature, and the resulting mixture dialyzed in the buffer (25 mmolar, pH 8.3) to provide the desired reagent.

Example 4: Preparation of Endometrial Antibody Capture Reagent

This is an example of the preparation of an endometrial antibody capture reagent of this invention.

A mixture of endometrial antigens (E_1, E_2, E_4 and E_5) were isolated as described in Example 1. The antigen fragments were further purified as follows:

Extraneous proteins were removed by precipitating them at 25% ammonium sulfate and centrifugation. The pellet was discarded, and the supernatant containing the antigen fragments was treated by adding ammonium sulfate to 40% and centrifugation, and the resulting pellet was resuspended in tris(hydroxymethyl)aminomethane buffer (pH 7.5). This suspension was dialyzed against buffer.

The antigens were was further purified by anion-exchange chromatography using a Waters PROTEIN-PAK™ DEAE column. The antigen fragments were then eluted from the column using tris(hydroxymethyl)amino-methane buffer (0.02 molar, pH 8) and a sodium chloride gradient. The antigens eluted at between 0.3 and 0.45 molar sodium chloride.

The resulting solution was diluted to 30 µg protein/ml in phosphate buffered saline solution containing protease inhibitors, and a sample (100 µl)

was added to each well of a polystyrene microtiter plate and incubated at room temperature for two hours. The plate was then washed three times with phosphate buffered saline solution. Remaining binding sites on the plates were blocked with bovine serum albumin (3%) in phosphate buffered saline solution for two hours at room temperature. The plate was then washed three times with a solution of TWEEN™ 20 nonionic surfactant (0.05%) in phosphate buffered saline solution.

Serum samples were diluted at 1:5 or 1:10 in a diluent of TWEEN™ 20 (0.05%) and bovine serum albumin (3%) in phosphate buffered saline solution. The diluted samples were incubated in the plate wells for two hours at room temperature while being shaken. The wells were washed three time with the wash solution containing TWEEN™ 20.

Goat anti-human immunoglobumin $F(ab')_2$ fragments conjugated to horseradish peroxidase, diluted in the diluent and filtered through a 0.2 micrometer filter, were added to the wells and allowed to react for 1 hour at room temperature.

The plate was again washed three times, and a dye-providing composition (200 µl) was added to each well and allowed to react for about five minutes. This composition included 2-(4-hydroxy-3-methoxyphenyl)-4,5-bis(4-methoxyphenyl)imidazole leuco dye (0.2 mmolar) poly(vinylpyrrolidone) (1.25 %), 4'-hydroxyacetanilide (5 mmolar), diethylenetriaminepentaacetic acid chelator (0.01 mmolar) and hydrogen peroxide (8 mmolar) in sodium phosphate buffer (10 mmolar, pH 6.8).

A solution (100 µl) to stop dye formation was added and the dye density was evaluated. Test samples were evaluated using a calibration curve generated from known positive and negative serum samples.

Example 5: Detection of Endometrial Antibodiesin Patient Specimens

This example demonstrates the use of the isolated endometrial antigens to detect endometrial antibodies present in patient blood sera by immunoblotting techniques.

The antigen fragments described in Example 1 were transferred to nitrocellulose strips using optimized immunoblotting techniques as described by Stott, supra. The buffer for transfer was composed of tris(hydroxymethyl)aminomethane buffer (25 mmolar, pH 8.3), glycine (200 mmolar) and methanol (20%, HPLC grade). To carry out the transfer, 75 volts at 4°C were applied for 2 hours. Upon completion of the transfer, nonspecific protein binding sites were blocked for one hour at 24°C with a "blocking" solution of tris(hydroxymethyl)aminomethane (20 mmolar, pH 7.5) containing gelatin (3%), normal goat serum (0.4%) and sodium chloride (500 mmolar). The blocked nitrocellulose was then washed twice, 5 minutes each time, with tris(hydroxymethyl)aminomethane (20 mmolar, pH 7.5) containing sodium chloride (500 mmolar) and TWEEN™ 20 nonionic surfactant (0.05%). The nitrocellulose strips and diluted serum samples (10 ml) were then contacted for incubation for two hours at 24°C. The serum had been diluted 100-fold in tris(hydroxymethyl)aminomethane (20 mmolar, pH 7.5) containing gelatin (1%), sodium chloride (500 mmolar), and TWEEN™ 20 nonionic surfactant (0.05%).

Sera believed to contain endometrial antibodies (as determined by indirect immunofluorescence microscopy) and as being from patients known to have endometriosis (as determined by laparoscopy) were tested. Sera known to be negative for endometrial antibodies using similar techniques were also tested as negative Controls. Sera was also tested without prior knowledge of the presence or absence of endometrisis ("blind" studies). The results

of these "blind" studies are noted in Table IV below by an asterisk (*).

After serum incubation, the nitrocellulose strips were washed four times (5 minutes each time) with the buffered solution containing TWEEN™ 20 noted above (30 ml) to remove uncomplexed materials.

The strips were then incubated for two hours at 24°C in contact with anti-human antibodies (13.2 µl of conjugate in 40 ml of solution used to dilute patient serum) directed to the serum endometrial antibodies bound to the immobilized transferred antigen fragments. The anti-human antibodies were comprised of goat anti-human IgG (heavy and light chain) antibodies labeled with alkaline phosphatase for detection. The conjugate was purchased as part of an IMMUN-BLOT™ assay kit (BioRad Laboratories). Upon completion of the antibody-conjugate incubation, the nitrocellulose strips were washed four times (5 minutes each) with the buffered TWEEN™ 20 solution noted above, and once (5 minutes) with the buffer solution noted above without TWEEN™ 20 to remove uncomplexed reactants and excess TWEEN™ 20 nonionic surfactant.

To detect the resulting bands in the strips, a dilute solution of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium [500 µl of each reagent stock in 50 ml of 0.1 molar tris(hydroxymethyl)aminomethane buffer, pH 9.5] were added.

Upon completion of sufficient color development of the bands (usually about 20 minutes), the substrate was removed and the strips were washed with deionized, distilled water for 10 minutes to quench further color formation. Tables III and IV below show the results of the sera screen using the assay noted above. The antigens used for obtaining the data in Table III were extracted using phosphate

buffered saline solution while Table IV data were obtained using antigens extracted using the buffered sucrose solution described in Example 1 above.

The FIGURE shows the immunoblot bands in strips 1a, 1b, 2a and 2b for the C_3 (40-44 kilodalton) and C_2 (33-37 kilodalton) fragments (identified in the FIGURE as "42 kD" and "35 kD", respectively). Bands 1c, 1d, 2c and 2d are negative controls and do not show bands for the noted fragments.

T A B L E III

Antigen Fragment(s)	Normal Patients (positives/ total samples)	Endometriosis Patients (positives/ total samples)
A	0/2	3/3
B_1	0/2	3/3
B_2	0/2	3/3
C_1	1/8	9/11
C_2	0/8	9/11
D_2	0/2	2/3

T A B L E IV

Antigen Fragment(s)	Normal Patients (positives/ total samples)	Endometriosis Patients (positives/ total samples)
C_2*	2/8	20/26
C_3*	2/8	19/26
C_4*	1/10	14/16
E_2*	0/5	5/5
E_3*	0/5	5/5
F_2	0/5	5/5
F_3	0/5	5/5
B_2	0/4	6/6
B_3	0/4	6/6
B_4	0/7	13/16

* "Blind" studies

Example 6: Detection of Endometrial Antibodies Using Disposable Test Device

This example demonstrates the detection of endometrial antibodies using a disposable test device and the ELISA immunological technique.

Materials:

Non-purified antigen (containing fragments C_1, C_2 and C_3, identified in Table I above) isolated from HEC1A cell line (as described above) was covalently attached to particles composed of poly[styrene-co-3-(p-vinylbenzyl-thio)propionic acid] (97.59:2.41 molar ratio, 1.4 mm average diameter) (2.53% solids) to form a particulate endometrial antibody capture reagent of this invention.

A particulate negative control reagent was prepared by similarly immobilizing α-casein on the same type of particles (2.05% solids).

The disposable test device used was a SURECELL™ test device (Eastman Kodak Company) having a LOPRODYNE™ polyamide microporous (5 µm) membrane (Pall Corporation) mounted therein. The test device has three test wells, one for the negative control, and two for the specimen. The particulate reagents described above (0.3% final solids) were coated on designated test well membranes in the test device, and dried overnight at room temperature.

The serum diluent composition used was composed of tris(hydroxymethyl)aminomethane (100 mmolar, pH 8.0), succinylated casein (1%), TWEEN™ 20 (0.05%) and gum arabic (1%) (available from Cetus Corporation). This diluent is similar to that described in EP-A-0 337 785 (noted above).

The wash solution was comprised of 1-methyl-2-pyrrolidinone (10%), NONIDET™ P-40 (0.1%), sodium chloride (500 mmolar), TWEEN™ 20 (0.25%), sodium phosphate, monobasic (50 mmolar, pH 7.4) (available from Cetus Corporation).

The anti-human antibodies used were goat anti-human IgG (heavy and light chain) antibodies and were conjugated with horseradish peroxidase (available from Jackson Immunoresearch).

The leuco dye composition comprised 2-(4-hydroxy-3-methoxyphenyl)-4,5-bis(4-methoxyphenyl)imidazole leuco dye (0.23 mmolar) poly(vinylpyrrolidone) (1.25 %), 4'-hydroxyacetanilide (5 mmolar), diethylene-triaminepentaacetic acid chelator (0.01 mmolar) and hydrogen peroxide (8 mmolar) in sodium phosphate buffer (10 mmolar, pH 6.8).

The dye stop solution comprised sodium azide (0.1%).

Assay Procedure:

A patient serum sample (25 µl) was diluted by adding it to the serum diluent (2 ml) in standard assay squeeze tubes. Filter tips were attached to the tubes, and the diluted sample was added to each well of the test device to the top of the fill dot. Fluid was allowed to flow through the membranes.

Diluted (1:10,000) labeled anti-human antibodies (40 µl) were added to each test well. Fluid was allowed to drain through the membranes and the test was incubated for one minute. Each test well was then washed twice (250 µl) with the wash solution.

The dye-providing composition (40 µl) was then added to each test well. The fluid was allowed to drain and the test was incubated for 2 minutes. The dye stop solution (80 µl) was then added, followed by fluid drainage.

The visual dye signal was evaluated and scored (0 being lowest dye signal and 10 being the highest). Transmission densities were also measured using standard densitometric procedures. The results are shown in Table V below.

TABLE V

	Sample 1*		Sample 2**	
	Negative Control Well (n=1)	Sample Test Well (n=2)	Negative Control Well (n=1)	Sample Test Well (n=2)
Transmission Density	0.02	0.057	0.015	0.023
Visual Results	1	5	0	2.5

* Serum Sample known to be positive for antibodies

** Serum Sample known to be negative for antibodies

"n" Identifies the number of test wells used in the assay, so that n=2 means that the results in the Table represent the average of two test well signals.

The invention has been described in detail with particular reference to preferred embodiments thereof, but it will be understood that variations and modifications can be effected within the spirit and scope of the invention. Moreover, all patents, patent applications (published or unpublished, foreign or domestic), literature references and other prior art referred to hereinabove are incorporated herein by reference for any teaching pertinent to the present invention.

We claim:

1. A protein antigen isolated from the cytoplasm of epithelial adenocarcinoma cells and selected from the group consisting of:

a. a fragment having a molecular weight of from about 63 to about 67 kilodaltons,

b. a fragment having a molecular weight of from about 33 to about 37 kilodaltons,

c. a fragment having a molecular weight of from about 40 to about 44 kilodaltons,

d. a fragment having a molecular weight of from about 31 to about 35 kilodaltons, and

e. a fragment having a molecular weight of from about 57 to about 64 kilodaltons.

2. The antigen of claim 1 wherein

said 63-67 kD fragment is isolated from the RL95-2, AN3CA, HEC1A, T47D or CAOV3 cell line,

said 33-37 kD fragment is isolated from the AN3CA, HEC1A, T47D or CAOV3 cell line,

said 40-44 fragment is isolated from the AN3CA, HEC1A, T47D or CAOV3 cell line,

said 31-35 fragment is isolated from the KLE cell line, and

said 57-64 fragment is isolated from the AN3CA, HEC1A, T47D or CAOV3 cell line.

3. A buffered antigenic composition useful for detecting the presence of endometrial antibodies, said composition comprising a protein antigen isolated from the cytoplasm of epithelial adenocarcinoma cells and selected from the group consisting of:

a. a fragment having a molecular weight of from about 63 to about 67 kilodaltons,

b. a fragment having a molecular weight of from about 33 to about 37 kilodaltons,

c. a fragment having a molecular weight of from about 40 to about 44 kilodaltons,

d. a fragment having a molecular weight of from about 31 to about 35 kilodaltons, and

e. a fragment having a molecular weight of from aboaut 57 to about 64 kilodaltons.

4. An endometrial antibody capture reagent comprising a protein antigen attached to a water insoluble support, said protein antigen isolated from the cytoplasm of epithelial adenocarcinoma cells and selected from the group consisting of:

a. a fragment having a molecular weight of from about 63 to about 67 kilodaltons,

b. a fragment having a molecular weight of from about 33 to about 37 kilodaltons,

c. a fragment having a molecular weight of from about 40 to about 44 kilodaltons,

d. a fragment having a molecular weight of from about 31 to about 35 kilodaltons, and

e. a fragment having a molecular weight of from about 57 to about 64 kilodaltons.

5. The reagent of claim 4 wherein said antigen is the 33-37 kD fragment, 57-64 kD fragment, the 40-44 kD fragment or a mixture thereof.

6. The reagent of claim 4 wherein said support is a microtiter plate.

7. The reagent of claim 4 wherein said support is a polymeric particle.

8. The reagent of claim 4 wherein said support is a microporous membrane.

9. A water soluble endometrial antibody reagent comprising a protein antigen which is detectably labeled, said protein antigen isolated from the cytoplasm of epithelial adenocarcinoma cells and selected from the group consisting of:

a. a fragment having a molecular weight of from about 63 to about 67 kilodaltons,

b. a fragment having a molecular weight of from about 33 to about 37 kilodaltons,

c. a fragment having a molecular weight of from about 40 to about 44 kilodaltons,

d. a fragment having a molecular weight of from about 31 to about 35 kilodaltons, and

e. a fragment having a molecular weight of from about 57 to about 64 kilodaltons.

10. The reagent of claim 9 which is labeled with a fluorogen, radioisotope or enzyme.

11. The reagent of claim 10 labeled with an enzyme selected from the group consisting of peroxidase and alkaline phosphatase.

12. A diagnostic test kit comprising:

1) a protein antigen isolated from the cytoplasm of epithelial adenocarcinoma cells and selected from the group consisting of:

a. a fragment having a molecular weight of from about 63 to about 67 kilodaltons,

b. a fragment having a molecular weight of from about 33 to about 37 kilodaltons,

c. a fragment having a molecular weight of from about 40 to about 44 kilodaltons,

d. a fragment having a molecular weight of from about 31 to about 35 kilodaltons, and

e. a fragment having a molecular weight of from about 57 to about 64 kilodaltons, and

2) an anti-human antibody reactive with an endometrial antibody.

13. The kit of claim 12 wherein said anti-human antibody is labeled with a fluorogen, radioisotope or enzyme.

14. The kit of claim 12 wherein said antigen is attached to a water insoluble support.

15. The kit of claim 12 further comprising a means for detecting the resulting reaction of said antigen with endometrial antibodies.

16. The kit of claim 12 further comprising a disposable test device comprising a microporous membrane.

17. A method for detecting endometrial antibodies comprising:

A. contacting a specimen suspected of containing endometrial antibodies with a protein antigen isolated from the cytoplasm of epithelial adenocarcinoma cells and selected from the group consisting of:

a. a fragment having a molecular weight of from about 63 to about 67 kilodaltons,

b. a fragment having a molecular weight of from about 33 to about 37 kilodaltons,

c. a fragment having a molecular weight of from about 40 to about 44 kilodaltons,

d. a fragment having a molecular weight of from about 31 to about 35 kilodaltons, and

e. a fragment having a molecular weight of from about 57 to about 64 kilodaltons, and

B. detecting any resulting complex of said antigen with said endometrial antibodies as an indication of said endometrial antibodies in said specimen.

18. The method of claim 17 wherein said antigen is attached to a water insoluble support as part of an endometrial antibody capture reagent, and the resulting complex is thereby insolubilized for detection.

19. The method of claim 18 wherein said support is a microtiter plate.

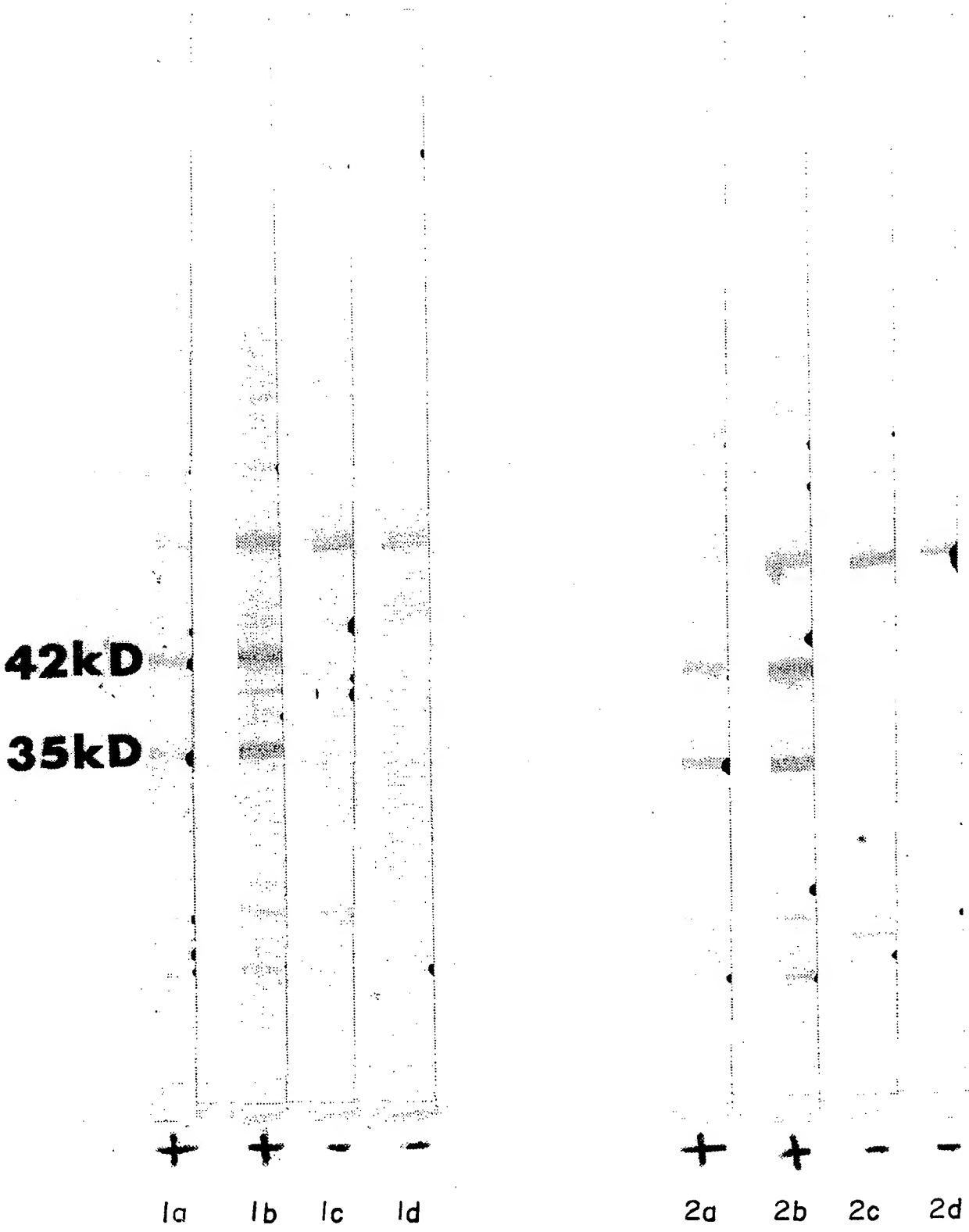
20. The method of claim 18 wherein said support is a polymeric particle.

21. The method of claim 17 wherein said method is carried out by detecting said complex by immunoblot.

22. The method of claim 17 wherein said complex is detected by reaction of said endometrial antibody with a detectably labeled anti-human antibody reactive with said endometrial antibody.

23. The method of claim 22 wherein said anti-human antibody is labeled with a fluorogen, radioisotope or enzyme.

24. The method of claim 17 wherein said specimen is blood serum.


42kD
35kD
+
+
-
-
1a
1b
1c
1d
+
+
-
-
2a
2b
2c
2d

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/02888

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C07K15/00; G01N33/68

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	G01N ; C07K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	EP,A,0 387 027 (ADEZA BIOMEDICAL CORPORATION) 12 September 1990 cited in the application see examples 16,17 ---	1-24
Y	AM. J. REPROD. IMMUNOL. MICROBIOL. vol. 13, 1987, pages 62 - 65; R. A. WILD ET AL.: 'Epithelial localization of antiendometrial antibodies' see page 63, column 1, line 46 - line 54; figure 1; table 2 ---	1-24
Y	FERTILITY AND STERILITY vol. 50, no. 6, 1988, pages 860 - 863; S. MATHUR ET AL.: 'Endometrial antigens' see page 861 - page 862; figure 1; tables 1,2 ---	1-24

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° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
13 AUGUST 1992	26. 08. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	SKELLY J.M.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	FERTILITY AND STERILITY vol. 55, no. 1, 1991, pages 90 - 94; R. A. WILD ET AL: 'Endometrial antibodies versus CA125'	

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9202888 SA 58899

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0387027	12-09-90	AU-A- 5068090	13-09-90
		CA-A- 2011704	07-09-90
		JP-A- 3202769	04-09-91

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82